



Contaminant accumulation and biological responses in Atlantic cod (*Gadus morhua*) caged at a capped waste disposal site in Kollevåg, Western Norway

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ABSTRACT

The aim of this study was to assess whether fish in Kollevåg, a sheltered bay on the western coast of Norway, previously utilized as a waste disposal site, could be affected by environmental contaminants leaking from the waste. Farmed, juvenile Atlantic cod (*Gadus morhua*) were caged for six weeks at three different locations in Kollevåg bay and at one reference location. Sediments and cod samples (bile and liver) were analyzed for polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), brominated flame retardants (BFRs), per- and polyfluoroalkyl substances (PFASs) and polycyclic aromatic hydrocarbon (PAH) metabolites, revealing a contamination gradient at the four stations. Furthermore, hepatosomatic index (HSI) and Fulton's condition factor (CF) were significantly lower in cod caged closest to the disposal site. Levels and activities of biomarker proteins, such as vitellogenin (Vtg), metallothionein (Mt), and biotransformation and oxidative stress enzymes, including cytochrome P450 1a and 3a (Cyp1a, Cyp3a), glutathione s-transferase (Gst) and catalase (Cat), were quantified in blood plasma and liver tissue. Hepatic Cat and Gst activities were significantly reduced in cod caged at the innermost stations in Kollevåg, indicating modulation of oxidative stress responses. However, these results contrasted with reduced hepatic lipid peroxidation. Significant increases in transcript levels were observed for genes involved in lipid metabolism (*fasn* and *acly*) in cod liver, while transcript levels of ovarian steroidogenic enzyme genes such as *p450scc*, *cyp19*, *3β-hsd* and *20β-hsd* showed significant station-dependent increases. Cyp1a and Vtg protein levels were however not significantly altered in cod caged in Kollevåg. Plasma levels of estradiol (E2) and testosterone (T) were determined by enzyme immunoassay (EIA) and showed elevated E2 levels, but only at the innermost station. We conclude that the bay of Kollevåg did not fulfill adequate environmental condition based on environmental quality standards (EQSs) for chemicals in coastal waters. Following a six weeks caging period, environmental contaminants accumulated in cod tissues and effects were observed on biomarker responses, especially those involved in reproductive processes in cod ovary.

1. Introduction

During the past century, oceans and coastlines were utilized deliberately for dumping of wastes (Goldberg, 1985). The bay of Kollevåg

outside the City of Bergen (Norway) was used as a waste disposal site from 1930 to 1975, with dumping of in total 450,000 m³ of industrial and household wastes. About 90% of this waste was deposited on the seabed (Vassenden and Johannessen, 2009). After terminating the

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Abbreviations

BaP	Benzo[a]pyrene
BNF	β -naphthoflavone
BFR	Brominated flame retardants
Cat	Catalase
CHL	Chlordanes
CF	Condition factor
CTD	Conductivity Temperature Depth
Cyp	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
EDC	Endocrine disrupting compounds
EIA	Enzyme immunoassay
EQS	Environmental quality standard
EROD	7-ethoxyresorufin-O-deethylase
E2	Estradiol
Gst	Glutathione S-transferase
Hsd	Hydroxysteroid dehydrogenase
HSI	Hepatosomatic index

HCH	Hexachlorocyclohexane
HPLC	High-performance liquid chromatography
HRGC	High-resolution gas chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MNE	Mean normalized expression
Mt	Metallothionein
OCP	Organochlorine pesticides
PFAS	Per- and polyfluoroalkyl substances
POP	Persistent organic pollutant
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PAH	Polycyclic aromatic hydrocarbons
Ssc	Side-chain cleavage
Star	Steroidogenic acute regulatory protein
T	Testosterone
TBARS	Thiobarbituric acid reactive substances
Vtg	Vitellogenin

dumping in 1975, the site was capped by sand and stone. Kollevåg was reopened as a recreational area in 1983 and has since been routinely monitored. In 2004, comparatively high levels of environmental contaminants were observed both in the sediments (e.g. polychlorinated biphenyls (PCBs): 271 $\mu\text{g}/\text{kg}$ dw of PCB-7) and in liver (PCB-7:

8679 $\mu\text{g}/\text{kg}$ ww) of cod captured in the area. Following this discovery, the sediment was covered with geotextile (Vassenden and Johannessen, 2009). After a few years of decreasing contaminant concentrations, increasing levels of PCBs were nonetheless observed in the sediments after 2009, both within and outside the capped area (Hatlen and

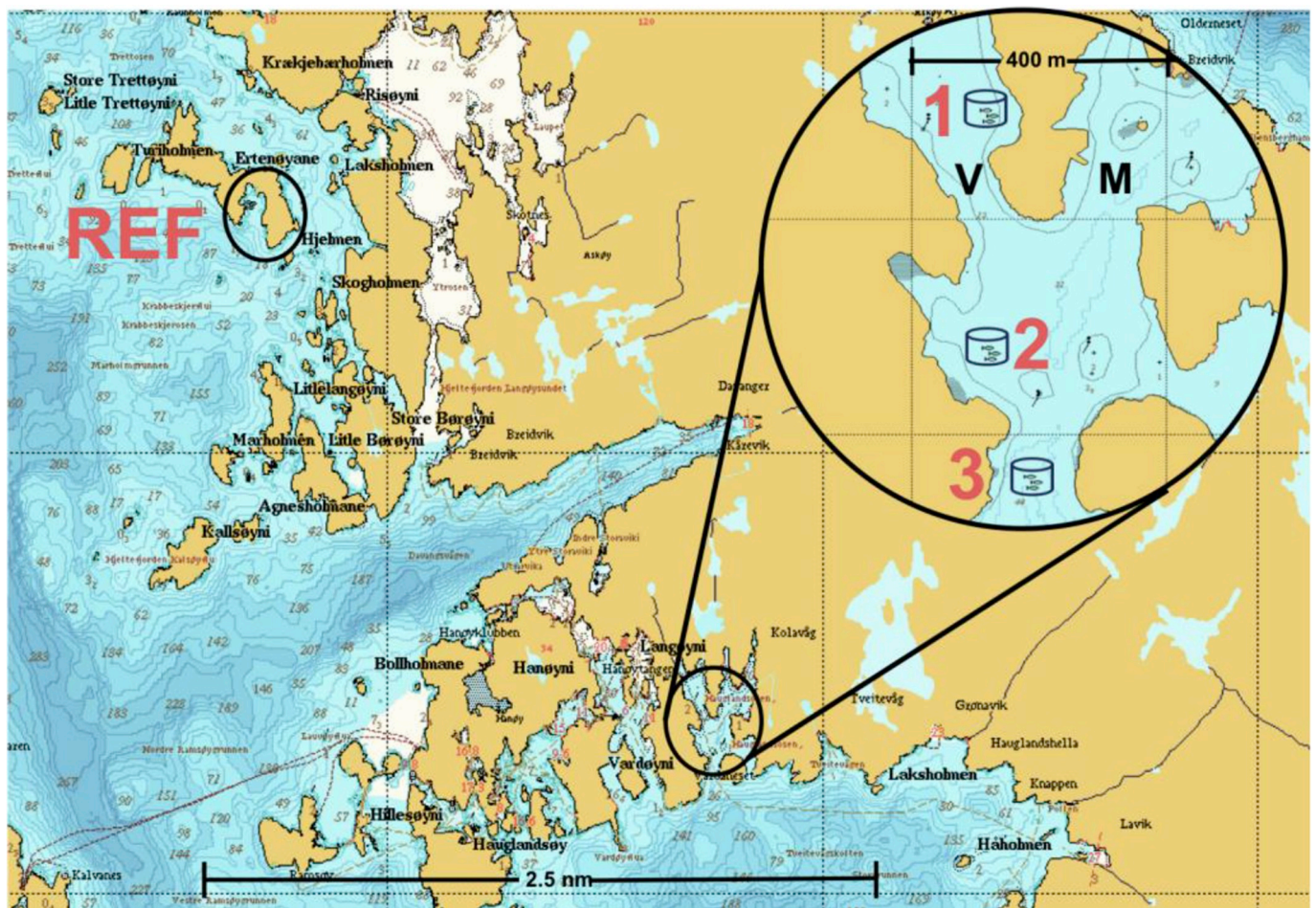


Fig. 1. Location of fish cages. Cages with 22 cod per cage were placed at four different locations: Three cages were placed in or near the capped waste disposal site in Kollevåg (enlarged in black circle), with one station (1) in the capped area in Vestrevågen (V), one station (2) at the borderline between Vestrevågen and Medavågen (M) and one station (3) at the outer parts of Kollevåg. In addition, a reference cage (REF) was placed near Ertenøyane. Nm = nautical miles. Map adapted from Olex AS.

Johansen, 2016). In 2014, extensive damage to the capping was observed and waste was visible on the surface (Hatlen et al., 2017). The area is still a popular beach and site for recreational fishing; it is therefore important to monitor environmental contaminant concentrations and biological effects on biota in the area.

PCBs, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs), and heavy metals, together with contaminants of emerging concern, such as endocrine disrupting compounds (EDCs), have been shown to affect the detoxification systems, lipid metabolism, antioxidant pathways and the endocrine system in fish (Arukwe and Goksøyr, 2003; El haimeur et al., 2017; Enerstvedt et al., 2018; Vincze et al., 2015; Yadetie et al., 2014). For example, EDCs are known for their potential to induce masculinization and feminization of female and male fish, respectively (Bergman et al., 2013). Furthermore, through disturbance of components involved in steroidogenesis, EDCs can cause effects on the whole organism level, potentially affecting sexual differentiation, growth, reproduction, and population structure (Arukwe, 2008; Kidd et al., 2007). In environmental toxicology, biomarkers such as cytochrome P450s (Cyps), vitellogenin (Vtg) and metallothionein (Mt) are used for monitoring purposes to indicate exposure to pollutants (Arukwe and Goksøyr, 2003; Hook et al., 2014; van der Oost et al., 2003).

The Atlantic cod (*Gadus morhua*) is a major fisheries species in the North Atlantic and is an important component of coastal and continental shelf ecosystems. It is commonly used as an indicator species in marine environmental monitoring programs, including the OSPAR convention and water column monitoring of offshore petroleum activities in Norway (Hylland et al., 2006; Norwegian Environment Agency, 2011; Vethaak et al., 2017). Cod has previously been used as a target species in caging studies to investigate pollution effects (Hylland et al., 2006). Caged fish have been utilized to study a wide variety of research questions including effects of wastewaters, environmental contaminants and seasonal changes (Berge et al., 2011; Beyer et al., 1996; Brammell et al., 2010; Chesman et al., 2007; Goksøyr et al., 1994; Vincze et al., 2015). Caging studies enable control of both animal placement and duration of exposure and most importantly, reflect exposure with natural background influences in a more realistic manner compared to laboratory experiments (Oikari, 2006).

Therefore, the aim of this study was to investigate whether environmental contaminants leaking from the waste disposal site in Kollevåg could accumulate and cause adverse biological responses in fish inhabiting the area, using caged cod as a proxy. Atlantic cod were caged at the site for a period of six weeks, and contaminant concentrations were determined in cod liver and in sediments, in addition to PAH metabolites in bile as a biomarker of PAH exposure. Biomarkers of effect were determined at transcript and protein levels in liver, ovaries and plasma to assess possible impacts on oxidative and bio-transformation stress responses, and reproductive processes.

2. Methods

2.1. Fish husbandry, caging and sampling

The experimental animals were juvenile, farmed Atlantic cod (mean weight 289.8 ± 73.2 g for all groups) that originated from Austevoll Research Station, Institute of Marine Research (IMR). Fish husbandry was performed in compliance to Norwegian animal welfare act and national regulations on the use of animal in research. The Norwegian Food Safety Authorities approved the experimental setup (Application number FOTS #9195) and the experiments were performed accordingly. Cod were given feed pellets up until two weeks prior to caging, after which pellets were gradually replaced with crustaceans (*Mysis* sp.). Cages (1.22 m^3) with 22 cod per cage were placed on the seabed at four different locations. Three stations were located in or near the capped waste disposal site in Kollevåg, Askøy, while one cage was placed at a reference location close to Ertenøyane (Fig. 1; Table S1).

Conductivity, temperature and depth (CTD) profiles taken at deployment and retrieval of the cages are found in Figs. S1–S4. The cod were caged for six weeks from early September to mid-October 2016. The cod were transferred to tanks on board R/V Hans Brattstrøm, and euthanized with a blow to the head. Tissue samples were collected and frozen in liquid nitrogen before being transferred to -80°C for downstream analyses. Regarding gonads, only female ovaries were collected due to the small size of male testis. For chemical analysis, liver tissue was stored at -20°C until analysis. Plasma samples were obtained from whole blood by centrifugation for 10 min at $1000 \text{ g} \times 4^\circ\text{C}$. Mucus was sampled using the TECO[®] Mucus collection set according to manufacturer's instructions (Product no. TE1034, Pathway diagnostics ltd, Dorking, UK).

2.2. Growth and condition parameters

Hepatosomatic index (HSI = liver mass (g)•100/body mass (g)) and Fulton's condition factor (CF = body mass (g)/body length (mm) •100,000) were used to evaluate the post-caging condition of the cod.

2.3. Chemical analyses

2.3.1. Sediment sampling and analyses

Sediment was collected from four spots at each station using a van Veen grab (KC Denmark, Silkeborg, Denmark). Sediment from the different spots were pooled to one sample, consisting of the upper 1 cm of the sediment layer, and was transferred to rilsan plastic bags (Tub-Ex ApS, Taars, Denmark). In order to not disturb the fish, sediments were sampled after recovery of the cages. The samples were frozen at -20°C for four months until elemental analyses with standardized methods at Eurofins Environment Testing Norway AS (accredited by the Norwegian Accreditation, NS-EN ISO/IEC 17025). These analyses included arsenic, lead, cadmium, copper, chromium, nickel and zinc according to NS EN ISO 17294-2; Mercury was analyzed with a modified version of EN ISO 17852; Loss of ignition and dry matter by NS 4764; PCB-7 according to EN 16167; PAH-16 by a modified version of ISO 18287 and grain size distribution by gravimetrics. The concentrations of metals, PCBs and PAHs were compared to the environmental quality standards (EQS) for organic contaminants in sediment according to EU legislation, summarized in the Norwegian water framework directive (Norwegian Environment Agency, 2018).

2.3.2. Cod liver analyses

2.3.2.1. OCPs, PCBs, BFRs and PFASs. The chemical analyses of persistent organic pollutants (POPs) and PFASs in cod livers were performed at the Laboratory of Environmental Toxicology, Norwegian University of Life Sciences (NMBU), Oslo, Norway. The laboratory is accredited by the Norwegian Accreditation for chemical analysis of OCPs, PCBs and BFRs in biota according to the requirements of the NS-EN ISO/IEC 17025 (TEST 137). PFAS analysis is not included in this accreditation, but it is validated according to the same procedures. POPs and PFASs were analyzed in eight pooled samples from each cage by assembling 2 g of cod liver from two fish of same sex in each pool (four pooled samples of each sex per cage). The analytical method for OCPs, PCBs and BFRs is based on Brevik (1978) and Polder et al. (2014), whereas the analytical procedure for PFASs is described by Grønnestad et al. (2017). The procedures are described in detail in supplementary material (S2).

2.3.2.2. Metals. Metals were analyzed in the pooled liver samples, i.e. the samples included in the chemical analyses described above, at the Faculty of Environmental Sciences and Natural Resource Management (MINA), Norwegian University of Life Sciences (NMBU). The liver samples were weighed, with approximately 500 mg, in ultrapure teflon tubes pre-rinsed in 7M HNO₃ and MQ water, before 5 mL HNO₃ (Ultrapure, subboiled) was added. As internal standards, Sc, Ge, Rh,

In, Bi and Au were added. Further, the samples were decomposed at 260 °C for 20 min in an UltraClave (Milestone Inc, Shelton, CT, USA). After digestion, the samples were diluted to 50.0 mL using distilled water in centrifuge tubes (Sarstedt, Nümbrecht, Germany). The elements were quantified with an Agilent 8900 ICP-MS-MS in oxygen reaction mode using standards for each element.

The analytical quality was approved by satisfactory quality control measures, and is further described in supplementary material (S2).

2.3.3. PAH metabolites in bile

Analysis of PAH metabolites in cod bile was performed at the Norwegian Institute for Water Research (NIVA). The method is previously described by Krahn et al. (1992) and in more detail in Kammann et al. (2013) (lab code 7). In brief, PAH metabolites were hydrolyzed with β -glucuronidase/arylsulphatase, separated with high performance liquid chromatography (HPLC) and detected by a fluorescence detector. Fluorescence was measured at the following wavelengths (nm, excitation/emissions): 1-OH-phenanthrene 256/380; 1-OH-pyrene 346/384; triphenylamine 300/360; 3-OH-benzo[a]pyrene 380/430).

2.4. RNA extraction and real-time-qPCR

2.4.1. Liver tissue

Liver tissue (100 mg) from Atlantic cod was homogenized with the Precellys 24 homogenizer by using ceramic beads CK28 (Bertin Technologies, Montigny-le Bretonneux, France). Total RNA was extracted using Agencourt[®] RNAdvance[™] Tissue Kit (Product no. A32645) on a Biomek NX^P workstation (Beckman Coulter Life Sciences, Indianapolis, IN, USA). The samples were treated with DNase according to manufacturer's instructions. RNA concentration, quality and integrity were checked using NanoDrop ND-1000 ultraviolet–visible (UV–Vis) spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA integrity number (RIN) of all samples were > 7. Transcript levels of target genes (*cyp1a*, *cat*, *gst3ab*, *fasn*, *acly*, *scd*, *hsp70*) and two reference genes (*uba52*, *ef1a*) (primer information in Table S3) were quantified using quantitative PCR (qPCR) as described by Olsvik et al. (2013), conducted according to the MIQE guidelines (Bustin et al., 2009). Mean normalized expression (MNE) of the target genes was determined using a normalization factor calculated by the geNorm software (Vandesompele et al., 2002). The geNorm stability index M for the reference genes was 0.73.

2.4.2. Gonad tissue

RNA was extracted from cod ovaries using Direct-zol[™] RNA extraction kit (Zymo Research Corporation Irvine, CA, USA), following the manufacturer's protocol. RNA quality was evaluated using NanoDrop ND-1000 ultraviolet–visible spectrophotometric analysis (Nanodrop Technologies) and formaldehyde agarose gel electrophoresis. cDNA was generated by following the instructions of iScript cDNA synthesis kit (Bio-Rad Laboratories Hercules, CA, USA). Primers specific for Atlantic cod *star*, *cyp17*, *p450scc*, *cyp19*, *3 β -hsd*, *11 β -hsd*, *17 β -hsd*, and *20 β -hsd* genes were amplified using iTaQ SYBR Green Supermix with ROX (Bio-Rad Laboratories) and Mx3000P real-time PCR machine (Stratagene, La Jolla, CA, USA). A detailed qPCR procedure and primer sequences are presented in the supplementary material (S3.2 and Table S4).

2.5. Liver protein analyses

Liver S12 fractions for various protein analyses were prepared as follows: Liver samples were kept on dry ice while cut and homogenized with a Precellys 24 homogenizer using 2.8 mm bulk zirconium oxide beads (Ref no. KT03961-1-102.BK, Bertin Technologies) in homogenization buffer as previously described (Nilsen et al., 1998). Post-

mitochondrial supernatant (S12) fractions were obtained by centrifugation for 20 min at 12,000 g \times 4 °C. Protein concentration of individual aliquots were determined using Pierce[™] 660 nm Protein Assay Reagent (Catalog no. 22660, ThermoFischer Scientific, Waltham, MA, USA). For the enzyme and TBARS assays, the results were normalized to the protein concentration of individual S12 fractions. All spectrophotometric and fluorometric measurements for the following liver analyses were performed using the 2300 EnSpire[™] Multilabel Reader (Perkin Elmer, Turku, Finland). Liver S12 fractions to be used as positive controls for Cyp1a induction were prepared by injecting Atlantic cod intraperitoneally with 50 mg/kg β -naphthoflavone (BNF), and euthanizing the cod one week after injection. However, the liver tissue preparation (homogenization and centrifugation) to obtain the S12 fractions were identical for positive controls and Kollevåg samples.

2.5.1. Western blotting

Liver S12 fractions were subjected to SDS-PAGE and western blotting according to Laemmli (1970) and Towbin et al. (1979), respectively. 15% polyacrylamide gels were utilized, and 7 μ g protein was loaded into each well. Primary antibodies utilized were anti-Cyp1a (1:800), anti-Cyp3a (1:800), anti-Mt (1:1000) and anti-actin (1:1000), and HRP-conjugated anti-IgG was used as secondary antibody (Table S5). Blots were developed with SuperSignal[™] West Femto Maximum Sensitivity Substrate (Item no. 34095, ThermoFischer Scientific) and visualized using the ChemiDoc[™] XRS + System (Bio-Rad Laboratories). For protein quantification, individual band intensities were divided by the intensity of the corresponding actin band using the Image Lab[™] Software (Bio-Rad Laboratories).

2.5.2. Antioxidant enzyme activity

The enzyme activities of Cat and Gst were analyzed using Catalase Assay Kit and Glutathione-S-transferase Assay Kit (Item no.707002 and item no. 703302, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The liver S12 fractions were diluted in the kit's separate sample buffers 1:2–3 and 1:80 for the Gst and Cat assays, respectively.

2.5.3. EROD activity

The 7-ethoxyresorufin O-deethylase (EROD) activity was determined in gills and in liver tissue. The EROD activity in liver was measured in S12 fractions as described in Burgeot et al. (1996), using the method of Burke and Mayer (1974), modified for plate readers by Galgani and Bocquene (1991). The EROD activity in gill filaments was performed essentially as described in Holth et al. (2014).

2.5.4. Lipid peroxidation (TBARS)

Lipid peroxidation was assessed in liver S12 fractions using the TBARS (TCA method) assay kit according to manufacturer's instructions (Item no. 700870, Cayman Chemical). The S12 fractions were diluted 1:2–10 in deionized H₂O prior to analyses. Samples were heated at 99 °C using a heating block (Eppendorf[®] Thermomixer[®] R, Sigma-Aldrich, St. Louis, MO, USA).

2.6. Quantification of plasma and mucus Vtg by ELISA

Levels of Vtg in blood plasma were determined both in male and female cod, using the Cod (*Gadus morhua*) Vitellogenin ELISA kit (Item no. V01006401-480, Biosense Laboratories AS, Bergen, Norway) according to manufacturer's instructions. The plasma samples were diluted 1:50 in dilution buffer before being analyzed. Levels of Vtg were also measured in mucus using the TECO[™] Multi-species Vitellogenin ELISA kit (Product No. TE1042, Pathway diagnostics ltd, Dorking, UK).

2.7. Steroid hormone analysis

Enzyme immunoassay (EIA) was used to determine the

concentration of 17 β -estradiol (E2) and testosterone (T) in plasma from female cod by using respective EIA kits (Item no. 582271 and Item no. 582701, Cayman Chemical). A detailed description of hormone extraction and quantification is presented in supplementary material (S5).

2.8. Statistics

Detection rate is defined as percentage of samples with a detectable value, i.e. above limit of detection (LOD). For POPs, PFASs and metals in liver, the compounds with detection rate below 50% within each cage are not included in further statistical analyses and only reported with range in Tables S8–S11. Compounds with detection rate above 50% within each cage are reported with descriptive statistics in Fig. 2 and Tables S8–11, the actual determined levels for samples below LOD are used, and missing values are replaced with the lowest determined level of the compound. For metals with detection rate above 50%, levels below LOD are replaced with $\frac{1}{2}$ LOD and levels between LOD and limit of quantification (LOQ) are replaced with $\frac{1}{2}$ LOQ.

For all results presented, Graphpad Prism 7 (Graphpad Software, San Diego, CA, USA) and R version 3.4.3 (R Core Team, 2017) were used for statistical analyses. Normality of the data was tested using Shapiro-Wilk. If one of the stations failed the Shapiro-Wilk test, the responses of all stations were log-transformed. Responses following

normal distribution were analyzed for station-dependent (Kollevåg stations vs reference station) statistical differences ($p < 0.05$) using a two-sample t -test with Welch Correction. Nonparametric tests (Mann-Whitney) were used to analyze responses failing Shapiro-Wilk after being log-transformed. In both tests, a two-sided alternative was used. To account for multiple testing, a Bonferroni corrected significance level $0.05/(3 \times 34) = 0.00049$ was applied in addition to the ordinary 0.05 significance level. Sediment results could not be analyzed statistically ($n = 1$).

To visualize the results, the investigated parameters were displayed in a spider plot (only reproduction parameters are shown). All values were rescaled to [0, 1] before calculating mean values by station. In addition, all determined parameters were combined in a principle component analysis (PCA). For the PCA, all missing data was imputed using the median values by station. The first two principle components and their eigenvectors are reported. In addition to a PCA, the relationship between all different parameters was visualized in a correlation plot. Pearson correlation was calculated and the parameters were plotted in a graph where parameters with a positive or negative correlation above 0.7 were connected. All parameters without any connections above this correlation cut-off were removed from the plot. The nodes were colored by parameter family (PCBs, OCPs, reproduction parameters, BFRs, liver transcripts, metals, PAHs, or PFASs).

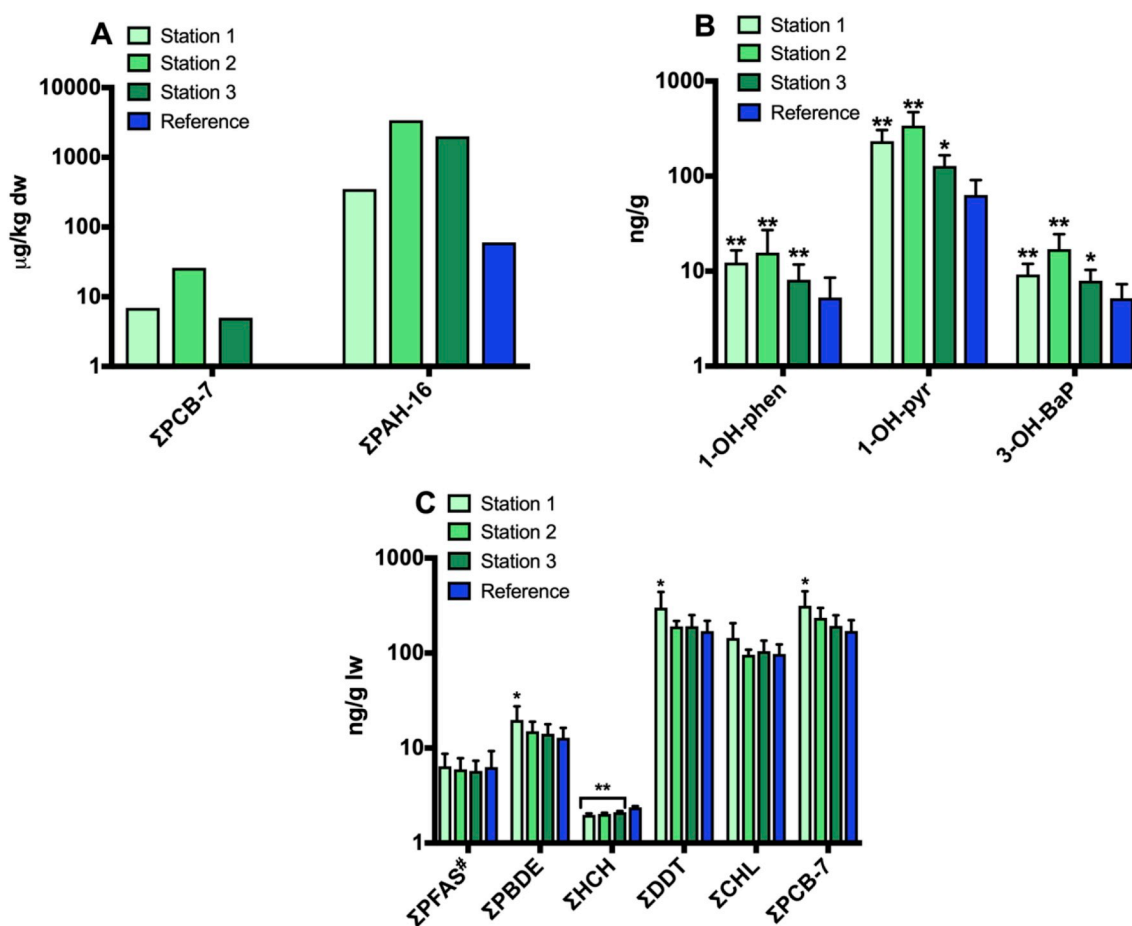


Fig. 2. Contaminant concentrations in sediments from caging areas and in liver and bile of Atlantic cod caged in Kollevåg. Sediment concentrations are presented as $\mu\text{g}/\text{kg}$ dry weight (dw) (A) and OH-metabolites of phenanthrene (1-OH-phen), pyrene (1-OH-pyr) and benzo(a)pyrene (3-OH-BaP) in bile (B) are presented as ng/g bile. In liver (C), contaminant concentrations are presented as ng/g lipid weight (lw), except $\Sigma\text{PFAS}\#$ which is expressed as ng/g wet weight $\#$. The y-axis is presented in log scale for all figures. For sediment analyses, four samples from each caging area were pooled prior to analyses (A). $n = 8$ for all stations in liver (C) measurements (two cod of the same sex were combined per n measurement) and $n = 19, 22, 16$ and 21 for stations 1, 2, 3 and reference stations in bile (B) measurements, respectively, data are presented as mean \pm s.d. $\Sigma\text{PCB-7}$ in sediments for the reference station were non-detectable. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. Double asterisk (**) indicates p -values below the Bonferroni corrected significance level ($p < 0.00049$).

3. Results

3.1. PCBs, PAHs and other contaminants were detected in sediments and cod tissues

The station-dependent differences in contaminant burdens in sediments are shown in Fig. 2A and Table S6. All Kollevåg stations had higher sediment concentrations of PCBs and PAHs, compared to the reference station. Σ PCB-7 exceeded EQS (4.1 $\mu\text{g}/\text{kg dw}$) for all three Kollevåg stations (6.9, 26 and 5.0 $\mu\text{g}/\text{kg dw}$ for station 1, 2 and 3, respectively). No EQS exists for Σ PAH-16 in the EU water framework directive, but several of the individual PAHs observed in sediments (anthracene, fluoranthene, pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[a]pyrene, indeno[1,2,3cd]pyrene, dibenzo[a,h]anthracene and benzo[ghj]perylene) exceeded their respective EQSs in one or several Kollevåg stations (Table S6). All Kollevåg stations had higher concentrations of all determined metals, except Cd, compared to the reference station, but the concentrations did not exceed the corresponding EQS (Table S6). The organic content within the sediments, measured as loss on ignition (LOI), were similar for the Kollevåg stations, but higher compared to the reference station (Table S7).

Selected hydroxylated PAH metabolites were determined in bile and the concentrations of selected contaminants including PCBs, DDTs, CHLs, PBDEs, PFASs and metals were determined in liver (detailed overview in Tables S8–S11). Significantly higher concentrations ($p < 0.05$) of the PAH metabolites 1-OH-phenanthrene, 1-OH-pyrene and 3-OH-benzo[a]pyrene were observed in cod bile from all Kollevåg stations compared to cod from the reference station (Fig. 2B). Station 2 had the highest concentrations for all three metabolites, followed by station 1 and station 3. In cod livers, Σ PCB-7, Σ DDTs and Σ PBDEs were significantly higher in cod from station 1, compared to the reference station (Fig. 2C). Cod from all Kollevåg stations had significantly lower levels of Σ HCHs compared to the reference ($p < 0.05$). No significant differences were found between stations for Σ PFASs, Σ CHLs and metals (Fig. 2C; Tables S8, S9 and S11).

3.2. CF and HSI were reduced in cod caged in the inner parts of Kollevåg

The parameters indicating growth performance, i.e. CF and HSI, are presented in Fig. 3. Compared to cod from the reference station, both CF and HSI were significantly reduced ($p < 0.05$) for cod from station 1, but not for the other Kollevåg stations (Fig. 3A and B).

3.3. Station-dependent differences in abundance of liver and ovarian gene transcripts

The transcript levels of selected genes were studied to elucidate any station-dependent differences in gene expression levels. Only a few significant differences ($p < 0.05$) in expression levels were observed

among the eight genes investigated in cod liver (Fig. 4A). Station 1 had a significantly higher expression level for *acly*, *fasn* and *gsta3b*. *Gsta3b* was significantly higher also for station 3, compared to the reference station. No significant changes were observed between the Kollevåg stations and the reference station for *cyp1a*, *cat*, *hsp70*, *fabp7* and *scd*. Among ovarian steroidogenic genes, significant increases ($p < 0.05$) were observed for *p450scc* and *20 β -hsd* in an apparent station-dependent manner (Fig. 4B). Station 1 and 2 had significantly higher expression level for *cyp17*, *cyp19* and *3 β -hsd*, while station 1 and 3 were highest for *17 β -hsd*. Compared to the reference site, *star* and *11 β -hsd* were significantly increased in station 2.

3.4. Cyp3a and Mt, but not Cyp1a, were detected in cod liver samples using protein immunoblotting

Western blots were performed to study levels of Cyp1a, Cyp3a and Mt in cod liver from the different stations. Cyp3a and Mt were detected in livers from all stations (Fig. 5) whereas Cyp1a was only detected in the positive control (Fig. 5A). When normalizing the band intensities of Cyp3a and Mt to the loading control (Actin), no significant differences in relative protein levels were seen for Cyp3a (Fig. 5B), however cod from station 1 and 2 had significant lower liver levels ($p < 0.05$) of Mt compared to the reference station (Fig. 5C).

3.5. No difference in Cyp1a (EROD) activity in cod caged at the different locations

EROD activities were assessed in both cod gill filaments and liver tissues, in order to reveal any changes in Cyp1a activity between the stations. In both tissues, very low activities ($< 10 \text{ pmol}/\text{min}/\text{mg}$ in liver, and close to, or below, the detection limit in gills), and no significant differences were observed when comparing the Kollevåg stations to the reference station (data not shown).

3.6. Reduced lipid peroxidation and antioxidant enzyme activities in Kollevåg stations

The enzymes Cat and Gst were studied to assess possible station-dependent differences in oxidative stress levels. The activities of both enzymes were significantly lower ($p < 0.05$) in cod from station 1 and 2, compared to cod from the reference station (Fig. 6A and B). Furthermore, the TBARS assay was performed to study the degree of lipid peroxidation and thereby possible oxidative stress in the cod livers. Compared to the reference station, cod from all Kollevåg stations had significantly lower hepatic concentrations ($p < 0.05$) of TBARS (Fig. 6).

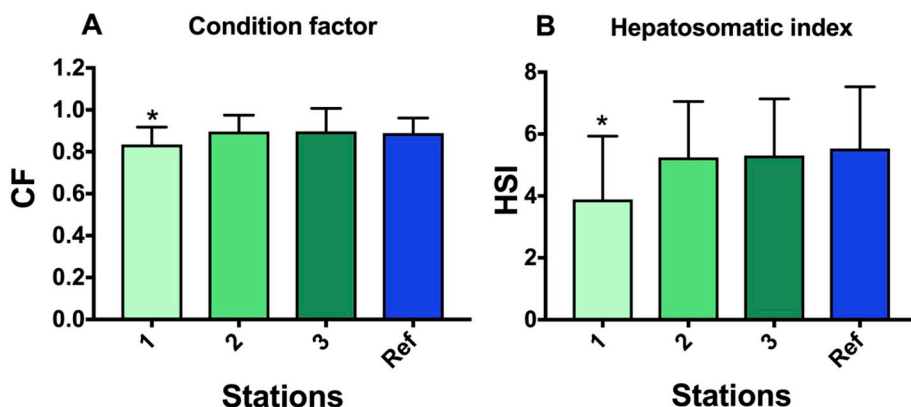


Fig. 3. Growth performance of caged Atlantic cod. Post-caging condition was assessed by calculating CF (A) and HSI (B). Data are presented as mean \pm s.d. $n = 21, 22, 16$ and 23 for stations 1, 2, 3 and Ref, respectively, asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. None of the responses had p -values below the Bonferroni corrected significance level ($p < 0.00049$).

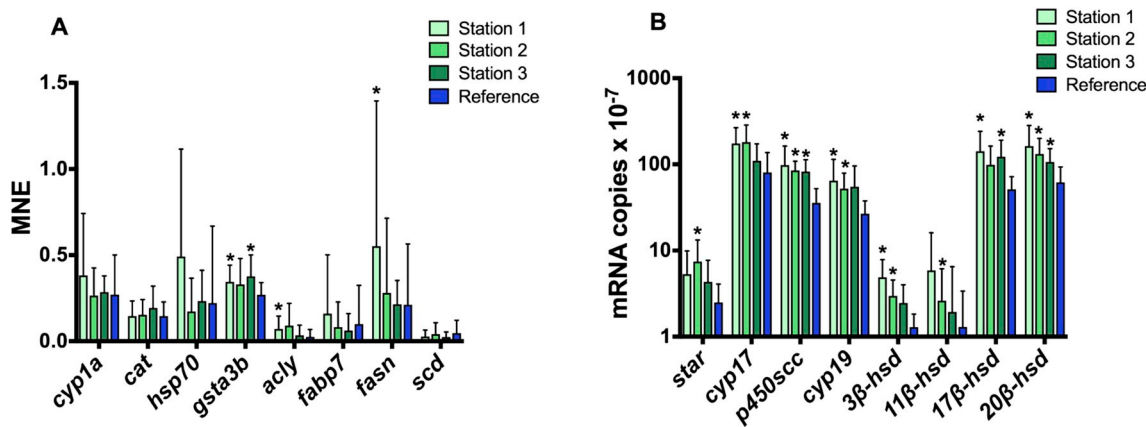


Fig. 4. Transcript levels of selected genes determined in liver and ovary samples from caged Atlantic cod. Quantitative PCR was performed on liver and ovary tissues from caged Atlantic cod. The expression levels are presented as mean normalized expression (MNE) for liver samples (A) and as transcript copies $\times 10^{-7}$ for ovary samples (B). $n = 12-15, 17-20, 13-15$ and $15-19$ for stations 1, 2, 3 and Ref, respectively (A). $n = 10, 7, 8$ and 9 for stations 1, 2, 3 and Ref, respectively (B). Data are presented as mean values \pm s.d. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. None of the responses had p-values below the Bonferroni corrected significance level ($p < 0.00049$).

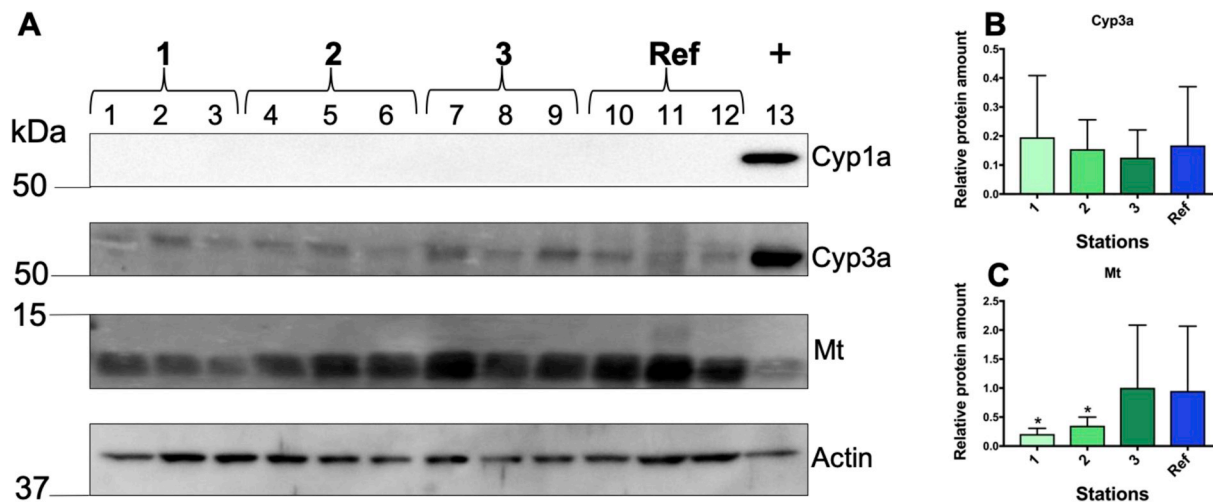


Fig. 5. Levels of Cyp1a, Cyp3a and Mt in liver S12 fractions of caged Atlantic cod. All samples were analyzed with western blotting and representative blots are shown in A and include the proteins Cyp1a, Cyp3a, Mt and Actin. The protein sizes are indicated on the left. Three samples representing individual fish from each station (1, 2, 3 and Ref in bold) are shown in A, 1–13 indicate well numbers. The positive control (+) in well no.13 is liver S12 fraction of cod exposed to 50 mg/kg BNF. For the protein quantification (B–C), individual band intensities were divided by the intensity of the corresponding actin band. $n = 18, 18, 12$ and 13 for stations 1, 2, 3 and Ref, respectively, and mean values \pm s.d. are presented in B–C. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. None of the responses had p-values below the Bonferroni corrected significance level ($p < 0.00049$).

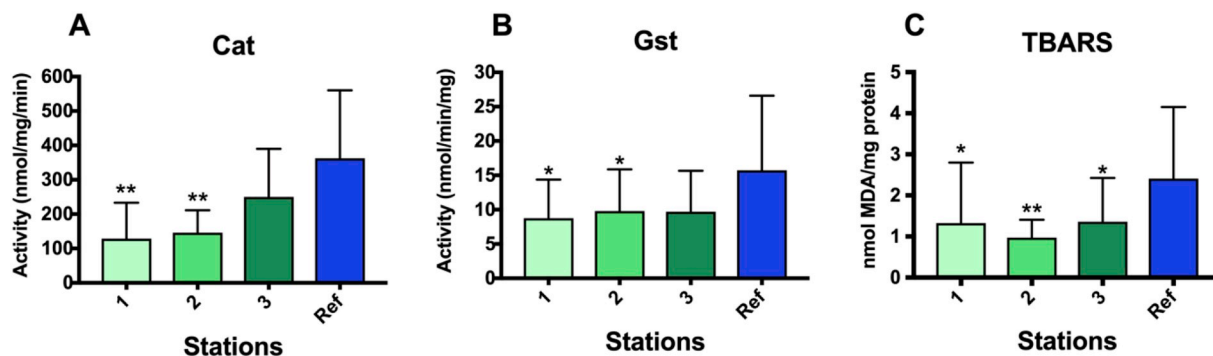


Fig. 6. Enzyme activities of Cat and Gst, and TBARS levels in liver S12 fractions of caged Atlantic cod. The enzyme activities of Cat (A) and Gst (B) and TBARS levels (C) were determined in cod liver S12 fractions. $n = 18, 20, 13$ and 17 (A), $n = 18, 21, 15$ and 20 (B) and $n = 21, 22, 16$ and 23 (C) for stations 1, 2, 3 and Ref, respectively. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. Double asterisk (**) indicates p-values below the Bonferroni corrected significance level ($p < 0.00049$).

3.7. Limited changes were observed in steroid hormones and Vtg levels in cod plasma

The plasma concentrations of E2 and T in female cod were determined using EIA. Cod from station 1 showed a significant increase ($p < 0.05$) in E2 concentrations compared to cod from the reference station (Fig. 7A), whereas no significant differences were found for T concentrations (Fig. 7B). Concentrations of Vtg were determined both in blood plasma and in mucus of male and female cod. No significant differences in plasma (Fig. 7C) or mucus (data not shown) Vtg concentrations were observed in comparisons among the stations or between males and females.

3.8. Combination of parameters show station-specific patterns and link biological responses to contaminant accumulation

The measured parameters involved in reproductive and steroidogenic pathways were combined in a spider plot to better visualize station-specific patterns (Fig. 8). The plot shows that the reference station is clearly separated from the Kollevåg stations regarding expression of steroidogenic enzyme genes. For steroid hormones and Vtg, this pattern is less clear, and only station 1 is markedly different from the reference station for E2 and T.

All chemical (individual compounds) and biological results except sediment data (due to $n = 1$) were combined in a principal component analysis (PCA) to clarify whether the contaminant levels and biological responses grouped in a station-dependent manner. In this analysis, the innermost stations of Kollevåg, stations 1 and 2, separated from the reference station, while station 3 was closer to and intersected with the 95% confidence ellipse of the reference station (Fig. 9A). Approximately 49% of the total variance was explained by the first two principal components (PCs). Using a Kruskal-Wallis-test, both PCs showed a statistically significant relationship to the stations ($p < 0.0001$) and Freeman's theta was higher for PC2 (0.757) than PC1 (0.643), indicating that PC2 captured more of the environmental differences between the stations. When studying the corresponding eigenvalues (Table S12), it was found that ovarian transcripts, bile PAHs, oxidative stress parameters, steroid hormones and some liver contaminants, explained most of the variability in PC2. The same chemical and biological data were subjected to a correlation analysis (Fig. 9B), revealing that among the biological data, the reproductive responses showed the highest correlations ($r \geq 0.70$) with liver contaminant levels. Using a lower correlation cut-off (< 0.70), the oxidative stress and condition parameters also correlated with the liver contaminant levels, whereas responses of liver transcripts, sex and Cyp3a seemed less correlated to other parameters (data not shown).

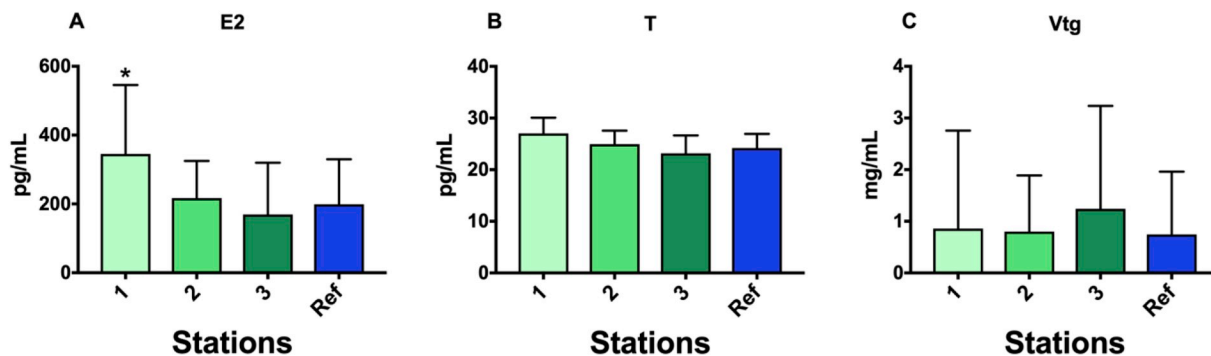


Fig. 7. Concentrations of estradiol, testosterone and vitellogenin in plasma of caged Atlantic cod. Estradiol (E2), testosterone (T), and vitellogenin (Vtg) concentrations (A, B and C, respectively) were determined in plasma samples. $n = 7, 6, 7, 9$ (A and B) and 19, 21, 16, 21 (C) for stations 1, 2, 3, Ref, respectively. Data are presented as mean \pm s.d. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. None of the responses had p -values below the Bonferroni corrected significance level ($p < 0.00049$).

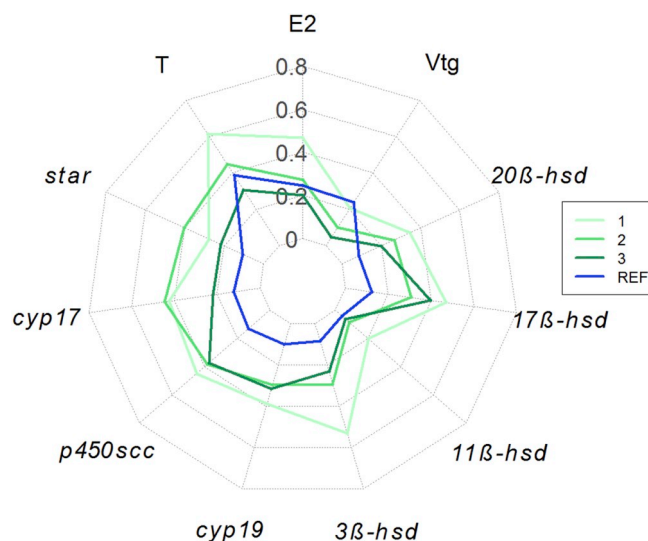


Fig. 8. Spider plot combining investigated reproduction parameters. The plot shows combined reproductive gene expression (italics) in ovaries and EIA (T, E2) and Vtg measurements in plasma of female cod. The values for all female fish were rescaled to [0, 1] and the mean values by station are shown.

4. Discussion

4.1. Contaminant concentrations in cod tissues reflect environmental concentrations

The present study investigated contaminant accumulation and biological responses of Atlantic cod caged for six weeks at a formerly capped waste disposal site in Kollevåg in Western Norway. We found that the sediment levels of contaminants, such as PAHs and PCBs, were higher in Kollevåg compared to the reference station (Fig. 2A). The main dumping site was located in Vestrevågen, close to station 1 in our study, whereas stations 2 and 3 were located further away from the original dump site (approx. 400–700 m), in an area that had not been capped. The results showed, however, that the sediments from station 2 contained the highest levels of PCBs and PAHs, and several of the compounds (e.g. BaP and pyrene) exceeded their respective EQSs (Norwegian Environment Agency, 2018). These findings are consistent with previous monitoring surveys in Kollevåg (Hatlen et al., 2013; Hatlen and Johansen, 2016), and might be explained by the location of station 2 at the borderline between two bays: Vestrevågen (where the capped area resides) and Medavågen (Fig. 1). Previously, elevated concentrations of both PCBs and PAHs have been detected in Medavågen, possibly from former local dumping activities. Thus, station 2

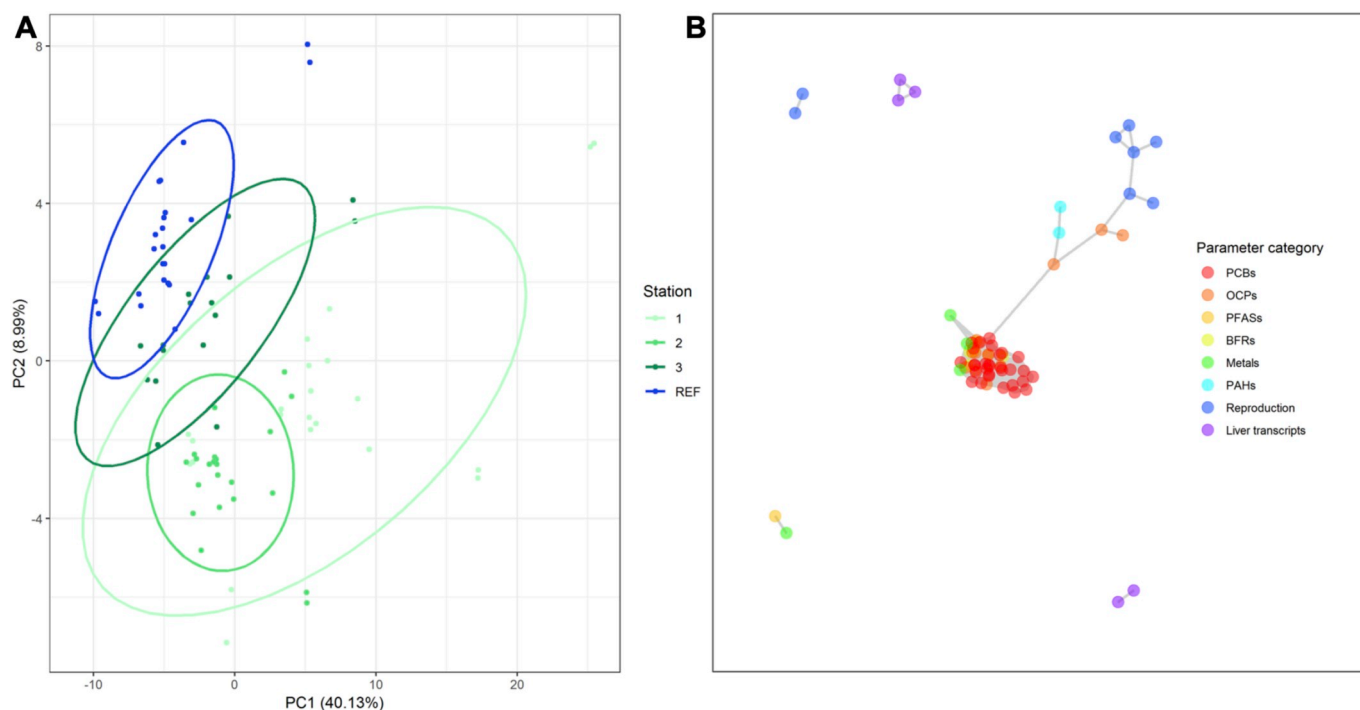


Fig. 9. Combination of measured parameters in principal component analysis (PCA) and correlation analysis. The data included is liver contaminant levels (individual compounds) and biliary PAH metabolites, condition parameters, qPCR data, oxidative stress analyses and parameters involved in steroidogenic and reproductive pathways. For the PCA (A), individual scores of the first and second principal component (PC) are plotted on an x-y scatter, giving a two-dimensional visualization of 49.12% of the variance. Each dot represents individual fish ($n = 84$). The ellipses are station-wise 95% confidence ellipses calculated from the first two principal components. The right panel (B) shows a correlation network where parameters which are correlated with a Pearson correlation above 0.7 or below -0.7 are connected. Each dot represents one parameter and parameters without connections were removed. The liver contaminants are grouped by PCBs, OCPs (including HCHs, CHLs and DDTs), BFRs (including PBDEs, HBB and HBCD), PFASs and metals, while reproduction includes ovarian genes, steroid hormones and Vtg.

may receive contaminant inputs from both bays (Hatlen and Johansen, 2016). Furthermore, drainage from Medavågen is also channeled into areas close to station 3, which may explain the similar and higher levels of PCBs and PAHs in sediments from station 3 compared to station 1. The present study shows that despite the capping of the main dump site in 2004, sediments within and outside the capped area in Kollevåg are still contaminated with PAHs and PCBs at concentrations of environmental health concern, with specific regards to long-term exposure and chronic toxic effects in the local fauna.

Bile PAH metabolites (in ng/g bile) from all Kollevåg stations and liver Σ 7PCBs, Σ DDTs and Σ 7PBDEs (in ng/g lipid weight) in cod from station 1, were significantly higher compared to cod from the reference station (Fig. 2). This demonstrates that caging of cod on the seabed for a six-week period resulted in accumulation of contaminants from the sediment, through water (gills and intestine) and/or through feeding on epibenthic organisms. The highest levels of bile PAH metabolites were observed in cod from station 2, which agrees with the pollution burdens observed in the sediments. Background and environmental assessment criteria (BAC/EAC) for phenanthrene and pyrene metabolites are 2.7/21 ng/g bile and 518/483 ng/g bile, respectively (Hylland et al., 2012). Three of the cod caged at station 2 exceeded EAC, indicating exposure of environmental concern. No assessment criteria have been developed for 3-OH-BaP, but the concentrations of this metabolite suggest elevated toxicological risk, since BaP is known to possess higher carcinogenic potential than many other PAHs (Delistraty, 1998). Liver PCB concentrations, however, were highest in station 1, while the sediment PCB concentrations were highest in station 2. This finding suggests that other factors, in addition to the sediment concentrations, can influence the exposure and contaminant accumulation in the caged cod, including water contaminant concentrations, the bioavailability of sediment-bound contaminants and accumulation in the local food web etc.

4.2. Condition parameters indicate health implications in cod caged at inner parts of Kollevåg

The condition parameters CF and HSI were significantly reduced for cod from station 1, compared to the reference station (Fig. 3). CF and HSI indicate energy status of the fish and a reduction can be caused by either food shortage, contaminant exposure and/or other factors (e.g. parasite infections) (Chellappa et al., 1995; Rätz and Lloret, 2003). Although Adeogun et al. (2016b) and Scarcia et al. (2014) attributed increased HSI and CF to contaminant exposure, PCB and other contaminant exposures are also reported to reduce condition parameters (Adeogun et al., 2016a; Ozmen et al., 2006; Scarcia et al., 2014). It is therefore uncertain whether the observed reductions in condition indices were caused by contaminant exposure or food shortage, or a combination of these.

4.3. Conflicting results among biomarkers of stress response, detoxification and oxidative stress responses in cod liver

We quantified the expression of genes involved in various biological pathways in cod liver (Fig. 4A). Increased expression of the lipogenic marker enzyme genes, *acly* and *fasn* might be related to the higher levels of PCBs detected in station 1, compared to the reference station. Indeed, increased mRNA of *acly* and *fasn* transcripts, and Acly protein levels have been observed in cod liver exposed to PCB153 (Yadette et al., 2017, 2014). These results are in accordance with other studies showing that PAHs and POPs, such as PCBs, are able to interfere with lipid metabolism (Grün and Blumberg, 2007; Ruzzin et al., 2010; Vieweg et al., 2018).

Cyp1a is an established biomarker for PAHs and dioxin-like PCBs (Brammell et al., 2010; Goksøyr, 1994; van der Oost et al., 2003; Whyte

et al., 2000). Surprisingly, despite our findings showing elevated levels of PAH metabolites in bile and dioxin-like PCBs in liver in cod from Kollevåg stations, Cyp1a was not significantly induced at neither the gene (Fig. 4A) nor protein level (Fig. 5), including enzymatic activity. Lack of Cyp1a induction may be explained by factors such as low concentrations of the inducers, or possible inhibitory effects of some components (e.g. fluoranthene) in the mixture (Billiard et al., 2004; Goksøyr et al., 1986; Wolińska et al., 2013; Brown et al., 2016; Hylland, 2006). Low inducibility and in some cases inhibition of Cyp1a activity in flounder (*Platichthys flesus*) exposed to selected PCB congeners has been reported (Besselink et al., 1998). Chronic contaminant exposure has also been shown to cause reduced Cyp1a inducibility in some fish species (Brammell et al., 2013).

In addition to Cyp1a, levels of Cyp3a and Mt were also assessed by immunoblotting. Whereas no significant differences in levels of Cyp3a were observed, a significant decrease in the levels of Mt were detected at station 1 and 2. The Mt decrease might be attributed to exposure to BaP and PCBs, contaminants previously shown to inhibit Mt synthesis and activity (Hurk et al., 2000; Maria and Bebianno, 2011; Sandvik et al., 1997).

Environmental contaminants can both induce and inhibit antioxidant enzyme activities (Benedetti et al., 2015; Scarcia et al., 2014) and reviewed in van der Oost et al. (2003). In the present study, hepatic activities of the antioxidant enzymes Gst and Cat were significantly reduced in the innermost stations of Kollevåg (Fig. 6). Benedetti et al. (2015) suggested that oxidative stress biomarkers can be induced in the initial phase of a response, followed by a depletion after longer periods. Other studies have indicated that an excessive ROS production can overwhelm the antioxidant system through direct interaction with enzymes, depletion of essential cofactors or substrates, or downregulation of corresponding transcripts (Dale et al., 2017; Shao et al., 2012). The reduction in enzyme activities could not be related to increased lipid peroxidation, as significant decreases in TBARS levels were observed in cod from stations 1 and 2. In accordance with our results, other studies suggested that there is no obvious link between antioxidant enzyme activities and lipid peroxidation (Oruç, 2010; Talcott et al., 1979).

4.4. Station-specific differences in biomarkers involved in reproductive pathways

To investigate possible endocrine disrupting effects, we analyzed the expression of a set of ovarian genes involved in the steroidogenic pathways (Fig. 4B). The gene encoding cytochrome P450 aromatase (*cyp19*), responsible for catalyzing the conversion of androgens to estrogens (Cheshenko et al., 2008), showed significantly higher expression levels at Kollevåg stations 1 and 2, compared to the reference station. Other genes including steroidogenic acute regulatory protein (*star*), cytochrome P450 side-chain cleavage (*p450scc*), 3 β -hydroxysteroid dehydrogenase (*3 β -hsd*), *cyp17*, *11 β -hsd*, *17 β -hsd* and *20 β -hsd*, which are also involved in the stepwise synthesis of sex hormones (progesterone, testosterone and estrogens) (Abbaszade et al., 1997; Adamski and Jakob, 2001; Kime and Ebrahimi, 1997; Miller et al., 1997) followed similar expression patterns as *cyp19*. In addition to the transcript results, it was shown that female cod from station 1 had significantly higher levels of circulatory E2 compared to the reference (Fig. 7). Albeit non-significant, a decreasing trend in the T level was observed across the stations. E2 and T play important roles in the development of female and male reproductive organs and control reproductive functions in adults. In accordance with our results, previous studies have shown that some PCBs, PBDEs and DDTs induce steroidogenesis, however, other congeners decrease the components of this pathway (Gregoraszczyk et al., 2008b, 2008a; Kraugerud et al., 2010; Murugesan et al., 2007; Song et al., 2008). Further, E2 binds to the estrogen receptor (ER) and induces the synthesis of Vtg, a precursor of yolk protein important for reproduction (Arukwe and Goksøyr, 2003; Tyler et al., 1996). Despite the higher E2 levels in cod at station 1, no

significant differences in Vtg levels were found between stations or sex. Previously, decrease or no change in the levels of Vtg was reported in female fish exposed to PCBs and PAHs in the wild (Casillas et al., 1991). Additionally, it is known that contaminants that bind and activate the ER can antagonize AhR pathway effects (Marques et al., 2013), while AhR agonists can antagonize estrogenic effects (Boverhof et al., 2008), resulting in ER-AhR cross-talk (Göttel et al., 2014; Mortensen and Arukwe, 2007). Complex contaminant interactions are important in understanding their effects on aquatic organisms as these may be synergistic, antagonistic or agonistic in nature (Celander, 2011; Silva et al., 2002). Considering the higher levels of PCBs, DDTs and PBDEs in cod liver from station 1 compared to the reference, the possible endocrine disrupting effects may be related to the complex mixture of environmental contaminants at the Kollevåg sites. Hence, these results may indicate possible effects on reproductive processes in feral fish inhabiting the area.

4.5. Combined chemical and biological parameters created a station-specific pattern

In order to investigate the pattern of all measured parameters in our study, we collected liver chemistry and bile PAH metabolite data of individual compounds and all biological responses in a PCA. Combining the contaminant concentrations and biomarker responses revealed a separation from inner to outer parts of Kollevåg, with station 3 grouping closest to the reference station, while station 1 and 2 were separated from the reference, using 95% confidence ellipses (Fig. 9A). To further identify any relationships between the contaminant levels and biological responses, all parameters were subjected to correlation analysis (Fig. 9B). Among the biological responses, parameters involved in steroidogenic pathways were found to strongly correlate to liver contaminant levels, suggesting a potential impact on reproductive pathways in the cod caged near the old waste disposal site in Kollevåg.

In addition to contaminant exposure, there are several factors that can contribute to the observed station-dependent differences. The complexity within field studies is evident – non-pollution-related confounding factors including individual biological variance (health, sex, metabolic activity, developmental and reproductive status) and environmental contributions (temperature, oxygen saturation, heterogeneity of environmental pollution) can contribute to observed effects (reviewed in van der Oost et al. (2003)). However, our analyses indicate strong correlation between contaminant levels and certain biological responses, suggesting that site-specific contaminant load is the main reason for the observed biomarker effects pointing to possible endocrine disruption.

4.6. Conclusion

In this study, we have shown that levels of PCBs and PAHs in sediments in Kollevåg exceeded the EQSs set in the EU water framework directive, and that these contaminants accumulated in cod tissues during the six-week caging period. Several biological responses were included in cod caged in Kollevåg compared to cod from the reference location, where reproductive endpoints were most prominent. Our results indicate that the area near the capped waste disposal site did not fulfill the requirements for the established environmental quality standards during our caging study, with a potential to affect reproductive processes in local aquatic organisms. The area surrounding station 1 was recapped during summer 2018, with an aim not only to improve conditions at station 1, but also the general environmental quality in the area. The caging strategy may be useful in follow-up studies to investigate the effect of the recapping of the Kollevåg waste disposal site.

Declarations of interests

AG is a major shareholder in Biosense Laboratories AS, supplier of Vtg ELISA kits.

Author contributions

MM, ZT, KH, FY, RLL, JLL, AA, KHy, OAK and AG were involved in experimental design and sampling planning. KD, MM, ZT, KH, FY, RLL, JLL, KHy, OAK, and AG contributed to sampling. KD performed liver qPCRs, enzyme activity assays (Gst, Cat, liver EROD), immunoblotting, vitellogenin and TBARS assays, in addition to contributing to statistical analyses. MM contributed to liver chemical analyses, and ZT performed laboratory analyses (EROD in gill filaments, biliary PAH metabolite extraction and data analysis). EK performed ovarian qPCRs and EIA measurements, and MG performed PAH metabolite analyses. HS and NB contributed with statistical and combination analyses. KD was the lead author, MM, ZT, EK, MG, NB, KH, RLL, and FY wrote parts of the manuscript, and all authors contributed to interpretation of the results and revision of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2019.02.003>.

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